9-1-2015

Developmental changes in hypoxic exposure and responses to anoxia in Drosophila melanogaster

Viviane Callier
Arizona State University

Steven C. Hand
Louisiana State University

Jacob B. Campbell
Arizona State University

Taylor Biddulph
Arizona State University

Jon F. Harrison
Arizona State University

Recommended Citation

Callier, V., Hand, S., Campbell, J., Biddulph, T., & Harrison, J. (2015). Developmental changes in hypoxic exposure and responses to anoxia in Drosophila melanogaster. *Journal of Experimental Biology, 218* (18), 2927-2934. https://doi.org/10.1242/jeb.125849

© 2015. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2015) 218, 2927-2934 doi:10.1242/jeb.125849

RESEARCH ARTICLE

Developmental changes in hypoxic exposure and responses to anoxia in *Drosophila melanogaster*

Viviane Callier[1], Steven C. Hand[2], Jacob B. Campbell[1], Taylor Biddulph[1] and Jon F. Harrison[1,*]

ABSTRACT

Holometabolous insects undergo dramatic morphological and physiological changes during ontogeny. In particular, the larvae of many holometabolous insects are specialized to feed in soil, water or dung, inside plant structures, or inside other organisms as parasites where they may commonly experience hypoxia or anoxia. In contrast, holometabolous adults usually are winged and live with access to air. Here, we show that larval *Drosophila melanogaster* experience severe hypoxia in their normal laboratory environments; third instar larvae feed by tunneling into a medium without usable oxygen. Larvae move strongly in anoxia for many minutes, while adults (like most other adult insects) are quickly paralyzed. Adults survive anoxia nearly an order of magnitude longer than larvae (LT50: 8.3 versus 1 h). Plausibly, the paralysis of adults is a programmed response to reduce ATP need and enhance survival. In support of that hypothesis, larvae produce lactate at 3× greater rates than adults in anoxia. However, when immobile in anoxia, larvae and adults are similarly able to decrease their metabolic rate, to about 3% of normoxic conditions. These data suggest that *Drosophila* larvae and adults have been differentially selected for behavioral and metabolic responses to anoxia, with larvae exhibiting vigorous escape behavior likely enabling release from viscous anoxic media to predictably normoxic air, while the paralysis behavior of adults maximizes their chances of surviving flooding events of unpredictable duration. Developmental remodeling of behavioral and metabolic strategies to hypoxia/anoxia is a previously unrecognized major attribute of holometabolism.

KEY WORDS: Ontogeny, P_{O_2}, Holometabolism, Metabolism

INTRODUCTION

Animals that undergo metamorphosis, including amphibians and many invertebrate groups, experience dramatic morphological and physiological changes during ontogeny (Mueller et al., 2015). These developmental changes enable animals to specialize their life stages for particular tasks; for example, holometabolous insect larvae are often specialized to feed, digest and grow on a particular diet, while the adults are often winged and specialized for mating and dispersal. The different habitats and locomotory capabilities of larval and adult holometabolous insects mean that they can experience very different oxygen environments and anoxic hazards. Many larval insects live in hypoxic conditions; for example, chironomid larvae often inhabit very hypoxic waters (Doke et al., 1995), *Cicindela* beetle larvae and *Ironoquia* caddisfly larvae live in sometimes water-logged soil (Cavallaro and Hoback, 2014; Hoback et al., 2000, 1998), and parasitic *Gastrophilus* larvae live in hypoxic horse intestines (Keilin and Wang, 1946). In contrast, the adult forms of these and most insects are terrestrial and likely only encounter anoxic or hypoxic conditions when trapped by water. These examples suggest that the ontogeny of holometabolous insects may be associated with dramatic shifts in the tolerance of hypoxia/anoxia and in their behavioral and metabolic strategies for coping with hypoxia/anoxia. Indeed, larval caddisflies that live in flood-prone wetlands are more than 10× more tolerant of immersion in hypoxic water than pupae (Cavallaro and Hoback, 2014).

Successful survival of anoxia is usually made possible by possession of metabolic systems that enable anaerobic ATP production to meet suppressed metabolic demand (Galli and Richards, 2014; Hochachka et al., 1996). Studies of insects that specialize on habitats low in oxygen such as eutrophic pools or water-logged soil have demonstrated that insects can extensively utilize anaerobic metabolism (Hoback et al., 2000; Kölsch et al., 2002). For larvae, chironomid larvae inhabiting hypoxic water are known to anaerobically convert glycogen to ethanol and acetate (Redecker and Zebe, 1988), the larval midge *Chaorobus crystallinus* anaerobically converts malate to succinate during anoxia (Englisch et al., 1982), the soil-living dipteran *Callitroga macellaria* larva anaerobically converts glycogen and amino acids to lactate, alanine and polyols (Gäde, 1985), and larval tiger beetles that experience flooding anaerobically produce lactate and alanine (Hoback et al., 2000). For adults, *Drosophila* are known to accumulate lactate, alanine and acetate (Feala et al., 2007), and anoxic locusts, honey bees, flies and beetles have been shown to accumulate lactate, alanine, glycerol, glycerol 3-phosphate and succinate (reviewed by Gäde, 1985; Wegener, 1987).

Suppression of metabolic demand is a key aspect of long-term survival of anoxia, as generally animals must rely on internal energy stores (Guppy and Withers, 1999; Hochachka et al., 1996). Vertebrates with good capacities to survive and down-regulate metabolism in anoxia such as turtles and goldfish can reduce their metabolic rates in anoxia to 10–30% of resting aerobic rates (Herbert and Jackson, 1985; van Ginneken and van den Thillart, 2009). Similarly, the marine priapulid worms *Sipunculus nudus* and *Halicryptus spinulosus*, the ghost shrimp *Lepidophthalmus louisianensis* and the bivalves *Astarte borealis* and *Arctica islandica* lower their metabolic rates to 20–60% of aerobic rates after 1 day or less of anoxia (Hardewig et al., 1991; Holman and Hand, 2009; Oeschger, 1990). Marine mollusks such as *Mytilus edulis* and *Acanthocardia tuberculata* reduce their metabolic rates even lower, to 5–6% of aerobic rates during multiple hours of anoxia (Theede, 1984; Theede et al., 1969). With multiple days of anoxia, anaerobic metabolic rates can fall to 1–2% of aerobic rates in some of these marine invertebrates (Oeschger, 1990; Oeschger et al., 1992). The animal champions of metabolic suppression during anoxia are the diapausing crustaceans (*Artemia*), which reduce

[1]School of Life Sciences, Arizona State University, Tempe, AZ 85287-4501, USA. [2]Department of Biological Sciences, Louisiana State University, Baton Rouge, LA 70803, USA.

*Author for correspondence (j.harrison@asu.edu)

Received 24 May 2015; Accepted 15 July 2015

Journal of Experimental Biology (2015) 218, 2927-2934 doi:10.1242/jeb.125849

metabolic rates to less than 0.5% of aerobic rates (Hand and Hardewig, 1996). The data available suggest that insects are relatively good at suppressing metabolic rate during oxygen limitation, which is a characteristic observed for other animals that have substantial capacities for surviving anoxia (cf. Hand, 1999). Species of tiger beetle larvae down-regulate their heat dissipation during anoxia to 3–14% normoxic rates (Hoback et al., 2000). Adult alder-leaf beetles reduce their energy flow in anoxia to 6% of that observed in normoxia (Kölsch et al., 2002), while locusts (*Locusta*) and *Manduca* caterpillars reduce heat dissipation rates during anoxia to 5% and 4% of aerobic rates, respectively (Moratzky et al., 1993).

Drosophila melanogaster are extremely well-developed genetic model organisms; because they share numerous genes and signaling pathways with humans, they are now being used to investigate many biomedical questions including several related to metabolism (Feala et al., 2009; Tennessen et al., 2011). However, many aspects of their behavior, 'lab ecology' and physiology remain poorly understood. For example, though it is recognized that *Drosophila* lab media has the potential to be hypoxic as a result of microbial activity, the actual oxygen levels in their lab and field habitats remain uninvestigated. Adult and larval *Drosophila* differ in their behavioral response to anoxia. Like most insects that have been studied, adult *Drosophila* are rapidly paralyzed (Krishnan et al., 1997). In contrast, larvae have been reported to exhibit an escape response to anoxia/hypoxia (Wingrove and O'Farrell, 1999), though their capacity to move in anoxia has not yet been quantified. Both adults and third instar larvae can tolerate reduced P_{O_2} down to approximately 1 kPa before exhibiting decreased activity and CO_2 emission rate, suggesting similar capacities to cope with hypoxia (Klok et al., 2010). Adult *Drosophila* have been reported to accumulate 10–15 nmol of lactate per fly in 1 h of anoxia (roughly 20 µmol g^{-1}; Vigne et al., 2009). Larval *Drosophila* exhibit elevated gene expression of lactate dehydrogenase and substantial lactate levels especially during the last portions of the third instar (Tennessen et al., 2011); however, to our knowledge, no study to date has been designed to compare the lactate-generating capacities of larval and adult *Drosophila* in anoxia. Here, we report that *Drosophila melanogaster* adults and larvae have dramatic differences in behavior, survival and lactate metabolism, and suggest that understanding such ontogenetic variation may be critical to understanding the physiological ecology of insects, genetic studies of metabolic regulation, and designing appropriate pesticide approaches.

RESULTS

Media P_{O_2}

Drosophila media, kept under normal laboratory conditions with relatively low egg densities, becomes extremely hypoxic within a few days after eggs are laid (Fig. 1A). With 50 eggs per vial, P_{O_2} declined slowly with media depth during the first 2 days after freshly laid eggs were placed into the vials; at this time, the larvae are in the first and second instar and remain near the surface, and so likely experience near-normoxic conditions. On days 3–4, when larvae are in the third instar and are tunneling up to 2 cm into the media, P_{O_2} declined precipitously to near 0 at depths of 0.3 mm and below (Fig. 1A). At higher egg densities, media P_{O_2} declined more rapidly with depth, and was below 8 kPa at 1 mm depth within 1 day of egg laying (Fig. 1B).

Locomotion in normoxia and anoxia

When placed onto the plate in normoxia, larvae exhibited exploratory behavior with a high rate (>20 min^{-1}) of locomotory undulations

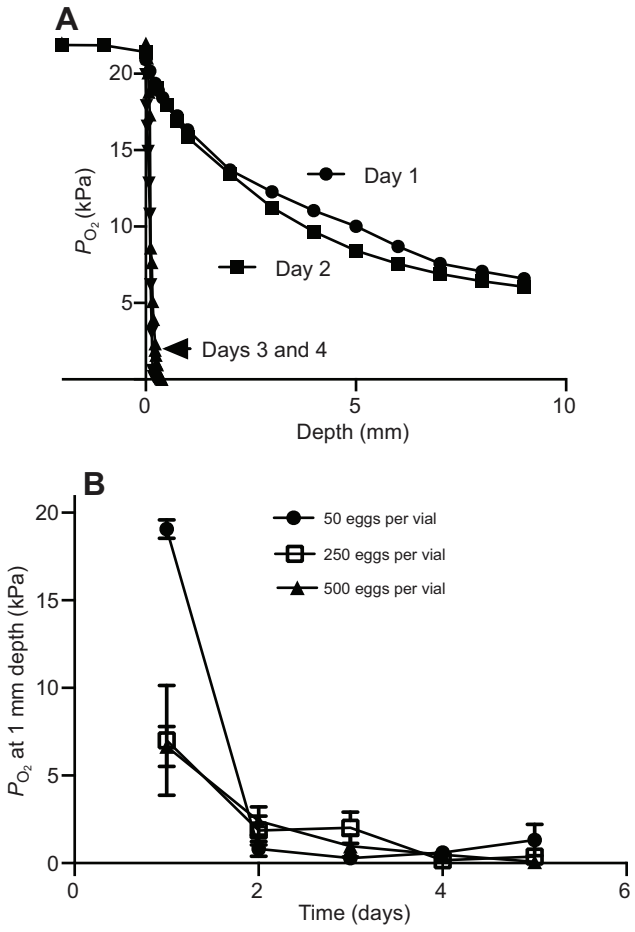

Fig. 1. Media P_{O_2}. (A) P_{O_2} at various distances from the media surface (0 mm depth) measured 1, 2, 3 and 4 days after egg collection for a 35 ml vial with 50 eggs. Above the media, P_{O_2} of the air was near 21 kPa regardless of day. On days 3 and 4, media P_{O_2} fell steeply with media depth (reaching values <0.2 kPa at 0.38 mm depth on day 3 and at 0.22 mm depth on day 4; day 3 and 4 data overlap each other). (B) P_{O_2} at 1 mm depth 1–5 days after egg collection for vials with 50, 250 and 500 eggs. For this and subsequent figures, mean and s.e.m. are shown.

(Fig. 2A,B). In approximately 5 min, larvae settled into feeding and began interspersing feeding and locomotion, with approximately10 locomotory undulations per minute (Fig. 2B). When exposed to anoxia, larvae ceased feeding, but for the first 5 min of anoxia, all larvae crawled at relatively high rates (Fig. 2A). For the subsequent 5–20 min of anoxia, the proportion of larvae moving remained high but their locomotion rates declined with time; thereafter, the proportion moving declined with time, and after 40 min of anoxia only a few of the larvae were moving during any minute of observation (Fig. 2A,B). The average duration of movement of larvae in anoxia was 38.8±4.35 min. All adults exhibited complete paralysis within 30 s of exposure to anoxia, with females being paralyzed more quickly than males (Fig. 2C; *t*-test, $t_{1,14}$=4.7, P=0.0003).

Survival

Adults survived approximately an order of magnitude longer than larvae under anoxia (Fig. 3; LT50 for adults≈8.3 h, for larvae≈1 h; Mantel–Cox test, χ^2=427, P<0.0001).

Lactate production

In normoxia, there was no difference in lactate concentrations between adults and larvae (pooled data: 4.7±0.22 mmol kg^{-1},

Journal of Experimental Biology (2015) 218, 2927-2934 doi:10.1242/jeb.125849

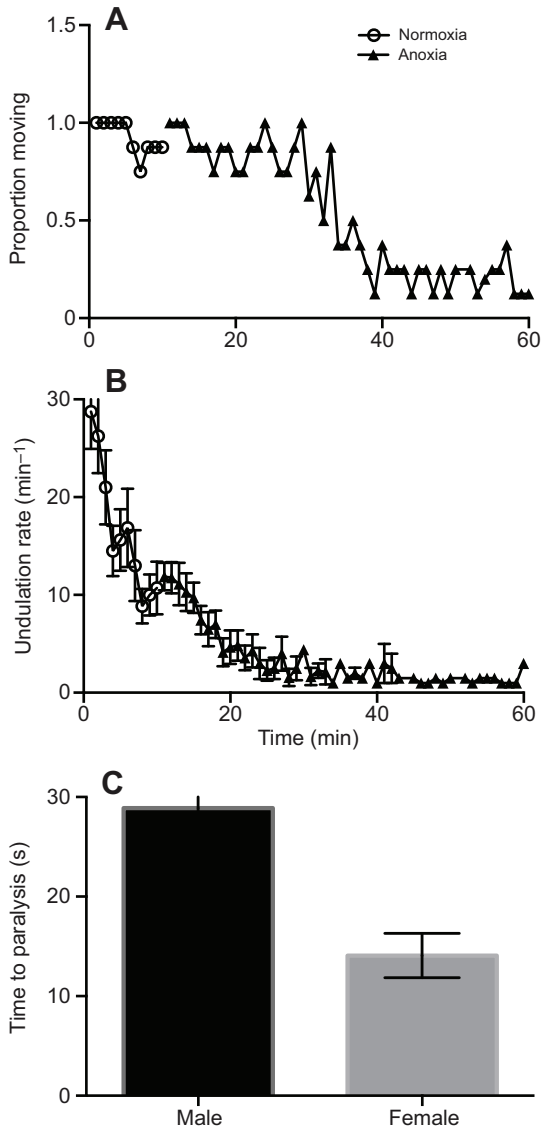

Fig. 2. Locomotion in normoxia and anoxia. The proportion of animals moving (A) and the number of locomotory undulations per minute (B) for late third instar larvae during 10 min of normoxia followed by 50 min of anoxia. (C) Time until paralysis after anoxia exposure in adults.

N=48). There was a small but significant decline in lactate levels from time zero to 90 min after handling (ANOVA, F=2.60, P=0.013). There was no significant interaction between stage and time; thus, adults and larvae did not differ in their patterns of lactate levels in normoxia.

In contrast, lactate levels increased strongly with time in anoxia for both larvae and adults (Fig. 4). There was a significant interaction effect between stage and time (general linear model, $F_{1,241}$=41.2, P<0.001), indicating that larvae accumulated lactate more rapidly.

Calorimetry

Heat dissipation rates of adults were high and fluctuating in normoxia, and fell dramatically to low, steady rates in anoxia (Fig. 5A). Groups of larvae showed similar patterns (raw data not shown). For all groups, we measured heat dissipation rates through 75 min of anoxia, during which time heat dissipation remained steady for both larvae and adults (Fig. 5B). For a few groups, we measured heat dissipation through 2 h of anoxia, during which heat

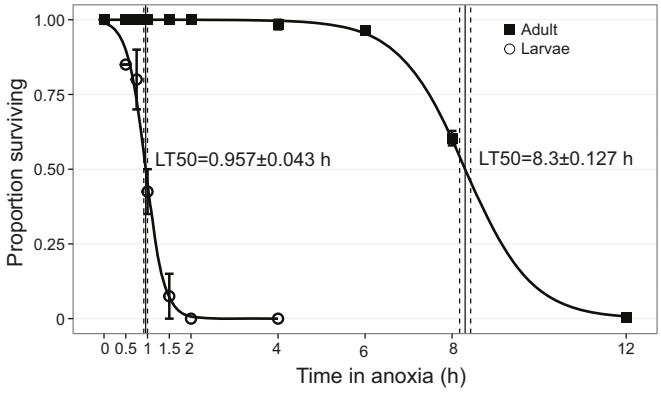

Fig. 3. Proportional survival of larvae versus adults. Survival is shown against time in anoxia for late third instar larvae and 3–4 day old adults.

dissipation rates also remained very steady (data not shown). Adults had higher mass-specific heat dissipation rates than larvae in normoxia (Table 1; $F_{1,8}$=22.6, P=0.0014), but adults and larvae did not differ in mass-specific heat dissipation rates in anoxia (Fig. 5B; P=0.9, pooled data: 0.43±0.113 mW g^{-1}). Adults and larvae had statistically similar suppression of metabolic rate in anoxia (t-test on arcsin-transformed data, P=0.69, anoxic heat dissipation/normoxic heat dissipation of the pooled data=0.026±0.0069). Heat dissipation calculated from lactate production during anoxia did not differ significantly from measured heat production in both adults and larvae (Table 1; Mann–Whitney U-test, P>0.2 in both cases); however, the power of this comparison was weak given the small sample size for the calorimetry data.

Normoxic gas exchange

Groups of adults had a significantly higher rate of O_2 consumption than larvae in normoxia (Table 1; ANOVA, $F_{1,38}$=16.6, P=0.0002), and lower respiratory quotients (Table 1; ANOVA, $F_{1,38}$=6.3, P=0.017). CO_2 emission rates were not significantly different between larvae and adults (Table 1; ANOVA, $F_{1,38}$=3.9, P=0.054). The average mass of adults was 0.71±0.039 mg, and the average mass of larvae was 1.36±0.054 mg (N=20 groups for each).

DISCUSSION

Holometabolism is well known to reorganize morphology and many aspects of physiology; this study demonstrates that behavioral and physiological strategies for responding to anoxia can also be dramatically altered by developmental stage in insects. This developmental plasticity provides a major degree of freedom for

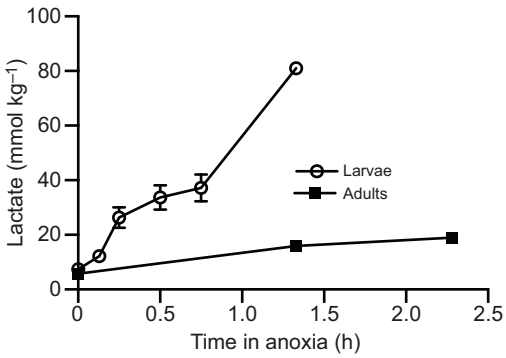

Fig. 4. Lactate concentration in anoxia. Lactate concentration is shown against time in anoxia for groups of adult and late third instar larval *Drosophila*.

Journal of Experimental Biology (2015) 218, 2927-2934 doi:10.1242/jeb.125849

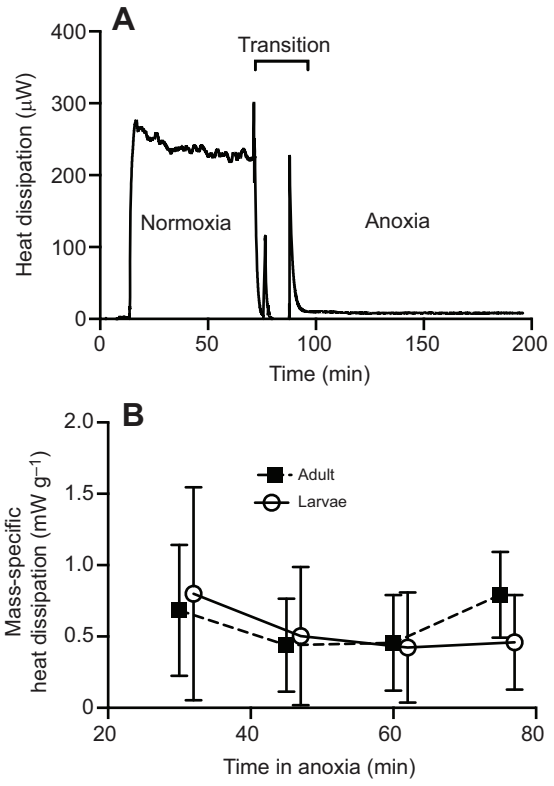

Fig. 5. Heat dissipation rate. (A) Sample plot of heat dissipation versus time for a group of *Drosophila* adults. The first 5 min show zero heat dissipation for an empty ampoule, followed by heat dissipation of flies in normoxia, and then anoxia. Data were discarded for the 20–30 min transition period during which the ampoule was removed from the calorimeter, flushed with argon, reinserted into the calorimeter, and allowed to reach thermal equilibrium. (B) Mass-specific heat dissipation during 20–75 min of anoxia exposure did not differ significantly between groups of larvae and adults.

animals to specialize different developmental stages to the environment. Similarly, strong developmental patterns in anoxia/hypoxia tolerance have been shown for many vertebrates, with vertebrate embryos often shown to be very anoxia resistant compared with adults (Podrabsky et al., 2007; Singer, 1999). In the case of *D. melanogaster*, the larvae live and feed in lab environments that pose severe anoxic hazards; a larva trapped during feeding with spiracles under the surface of the media will be unable to obtain oxygen. Thus, a *Drosophila* larva tunneling into its media to feed is rather like a diving seal, venturing into a liquid media with no options for obtaining oxygen until it returns to the surface. If the vial is crowded and overly moist, the media can become a viscous anoxic gel that can trap the larvae. As *Drosophila* larvae live in rotting plant material in nature, it is likely that such hypoxic threats also occur naturally. Plausibly, such habitats have selected for the behavioral strategy of exhibiting strong and prolonged escape locomotion out of anoxia, a response not seen in adult *Drosophila* or larval insects that experience non-escapable anoxia such as flooded borrows (Hoback et al., 1998).

Table 1 . Metabolism parameters calculated for animals in normoxia and after 20-75 min of anoxia

	Larva	Adult
Normoxia		
O_2 consumption rate (μmol g^{-1} h^{-1})	87.1±7.50*	127.2±6.42
CO_2 emission rate (μmol g^{-1} h^{-1})	71.4±8.12	91.1±5.45
Respiratory quotient	0.81±0.025*	0.710±0.028
Heat dissipation calculated from oxygen consumption (mW g^{-1})	10.9±0.93	15.9±0.80
Calorimetrically measured heat dissipation (mW g^{-1})	14.6±0.99*	18.1±0.99
Anoxia		
Lactate accumulation rate (μmol g^{-1} h^{-1})	20.0 +/–*	7.2 (+/–)
Heat dissipation calculated from lactate production (mW g^{-1})	0.44	0.16
Calorimetrically measured heat dissipation (mW g^{-1})	0.45±0.369	0.45±0.306

Means±95% confidence limits are presented. An asterisk indicates a significant difference between larvae and adults (*t*-tests).

While there are few direct comparisons of larval and adult insect physiological responses to anoxia in the literature, the data available suggest that dramatic differences between larvae and adults are common; however, which stage is more anoxia tolerant varies. As noted above, larval caddisflies are more than 10× more tolerant of immersion in hypoxic water than pupae (Cavallaro and Hoback, 2014); similarly, larval tiger beetles are able to tolerate approximately 3× longer anoxia durations compared with adults (Brust and Hoback, 2009). Conversely, in the staphylinid intertidal beetle *Bledius spectabilis*, the duration of anoxia that could be survived by the median adult was 50 h versus only 16 h for larvae, while just-hatched larvae survived only a few hours (Wyatt, 1986). In our experiments, adults were able to survive approximately an order of magnitude longer duration of anoxia than larvae. These data suggest that developmental remodeling of tolerance to hypoxia/anoxia is widespread, and that the direction of variation may vary with the ecology of the insect.

The mechanistic basis for the much greater tolerance of adult relative to larval *Drosophila* remains unclear. The differences in behavioral responses, with adults exhibiting rapid paralysis while the larvae exhibit vigorous escape behavior, may have consequences that explain the better capacities of adults to survive anoxia relative to larvae. Plausibly, the higher rates of lactate accumulation in the larvae cause changes in intracellular pH that affect protein structure, compromise homeostasis and lead to more rapid death from anoxia. While we were not able to measure heat dissipation during the first 20–30 min of anoxia, it seems likely that larval metabolic rates are three times higher than adults' during this initial period of anoxic exposure based on lactate production rates and behavior; adult and larval metabolic rates did not vary during longer term anoxia (Fig. 5B). A higher rate of metabolism during initial anoxic exposure could drive more rapid depletion of high-energy phosphates, leading to downstream effects of depleted ATP on mitochondrial and cellular homeostasis (Galli and Richards, 2014). Additionally, higher rates of metabolism during the initial 20 min of anoxia could cause larvae to more rapidly deplete glycogen stores.

The rate of lactate accumulation (approximately 60 mmol kg^{-1} h^{-1}) in these anoxic, escaping *Drosophila* larvae is among the highest reported for any insect or animal in anoxia. Some turtles and carp are paradigms for anoxia tolerance in vertebrates. In carp, lactate accumulation is approximately 0.72 mmol kg^{-1} h^{-1} (Johnston and Bernard, 1983). In turtles, plasma lactate can accumulate to 150–200 mmol l^{-1}, but this requires 3–5 months of anoxia at 3°C (Jackson, 1997). Ghost shrimp, which live in marine sediments and are highly tolerant of anoxia, accumulate 120 mmol lactate kg^{-1} in about 50 h, a lactate accumulation rate 1/25 that of anoxic *Drosophila* larvae (Holman and Hand, 2009). The only comparable rate of lactate formation during anoxia ever reported for animals was for *Culex pipiens* mosquito larvae, which accumulated lactate at a rate of about

Journal of Experimental Biology (2015) 218, 2927-2934 doi:10.1242/jeb.125849

55 mmol kg^{-1} h^{-1} (measured over a period of 1.5 h) (Redecker and Zebe, 1988), a value quite similar to that shown here for *Drosophila* larvae. The rates of lactate accumulation at the whole-body level in late third instar *Drosophila* larvae in anoxia is higher than the highest rates measured during burst activity in insects; grasshoppers accumulate lactate at 300 mmol kg^{-1} leg muscle h^{-1} during jumping, which is approximately 22 mmol kg^{-1} whole-body mass h^{-1} (Harrison et al., 1991), but lower than that measured during burst activity for some other animals. For example, during 5 min of burst, exhaustive activity, the lizard *Sceloporus occidentalis* accumulates 228 mmol kg^{-1} whole-body mass h^{-1} (Bennett and Gleeson, 1976), and rainbow trout over 150 mmol kg^{-1} whole-body mass h^{-1} (Milligan and Girard, 1993). The decline in lactate production rates in adults is consistent with the approximately twofold decrease in anoxic lactate production rates observed from the beginning to the end of the pupal period in flies (Merkey et al., 2011).

Another interesting aspect of holometabolic biochemical reorganization may be changes in pathways for anaerobic metabolism. In larvae, lactate production can explain 100% of heat dissipation, but in adults, it explains only about 33% of heat dissipation (Table 1). *Drosophila* adults accumulate considerable alanine and acetate in addition to lactate during severe hypoxia (Feala et al., 2007), probably improving ATP formation from glycogen (Feala et al., 2009) and enhancing the anoxia tolerance of adults relative to larvae.

Recently, some authors have suggested that the fast-growing *Drosophila* larvae exhibit aerobic lactate production (aerobic glycolysis) as a mechanism to produce additional metabolites for synthetic pathways, as has been reported for cancer cells and some cell culture lines (Locasale and Cantley, 2010; Tennessen et al., 2011; Vander Heiden et al., 2009). While this may be true, our data demonstrating that *Drosophila* media is strongly hypoxic suggest that the lactate production capacity in *Drosophila* larvae can be explained by the need to move and feed in hypoxic media. The estimated rate of heat generation of larvae based on oxygen consumption was only 75% of that measured by calorimetry (Table 1). However, because these estimates used different groups of animals and conditions, it is plausible that this difference related to variation in activity levels. For aerobic glycolysis to contribute significantly to proliferative growth, catabolism of fuels (exogenous or stored) must be associated with transfer of significant carbon moieties from these fuels to biomass. Stable isotope analyses should enable a rigorous test of this hypothesis.

The anoxia tolerance of adult *Drosophila* is not exceptional for insects. A recent meta-analysis found that the mean duration of anoxia that could be survived by insects was 35 h, with an average of 9 h for species from normoxic habitats and an average of 80 h for those in danger of being trapped in anoxic conditions (Harrison, 2015). In contrast, the duration of anoxia that can be survived by 50% of *Drosophila* larvae was 1.2 h, shorter than that reported for any other insect larvae to date, except for just-hatched staphylinid beetles (Harrison, 2015). These data suggest that exposure to viscous anoxic media may have selected for *Drosophila* larvae to exhibit high rates of escape locomotion that compromise anoxic survival.

Both adult and larval *Drosophila* were able to suppress heat dissipation rates to 2.5% of normoxic rates during 0.5–2 h of anoxia (Fig. 5). This is a much greater capacity to suppress metabolism than is seen for most vertebrates, and is also greater than has been observed in most other insects (see references in the Introduction). For example, in frogs, heat dissipation decreases to 2/3 of its normoxic value after 30 min of anoxia, and eventually decreases to 20% of the normoxic value when anoxia is maintained beyond 30 min (Wegener, 1988); in turtles, metabolic rate estimated from lactate accumulation drops to 18% of normoxic values after 8 h of anoxia (Herbert and Jackson, 1985). Thus, this genetic model organism is revealed as an excellent model organism for studying possible mechanisms for this metabolic suppression; plausible mechanisms include channel arrest and growth suppression. *Drosophila* have already been used to identify multiple key pathways by which anoxia blocks the cell cycle and growth (DiGregorio et al., 2001; Douglas et al., 2005, 2001; Foe and Alberts, 1985).

MATERIALS AND METHODS

Animals and general conditions

For all experiments we used the Samarkand strain of *D. melanogaster*. Flies were maintained in culture at Arizona State University on cornmeal and molasses diet (Stieper et al., 2008) at 25°C in 300 ml plastic bottles. Each bottle contained no more than 200 eggs, to avoid overcrowding. From these bottles, late third instar larvae were chosen, using the criteria of lack of food in the gut (larvae were placed on dyed food to allow visualization of the gut), being found wandering on the vial walls, or weighing more than 1.2 mg. Anoxia tolerance varies with age in adult *Drosophila* (Benasayag-Meszaros et al., 2015), so we only used 2–4 day old adults; these were obtained by clearing bottles of all adults, collecting adults over a 1 day period, and then transferring these adults (without anesthesia) to new vials. All experiments were run at 25±1°C.

Measurement of media P_{O_2}

At least 1000 adults were placed into a 4 l flight chamber; Petri dishes with grape agar and yeast paste were placed into the cage and eggs were collected over 3 h periods. Eggs were transferred into 35 ml plastic vials filled with 2 cm of food, with 50, 250 or 500 eggs per vial, with five vials per density. Approximately 1 day later, and then each day until the larvae began to pupariate, we measured the media P_{O_2}. We used a Presens (Regensburg, Germany) Micro TX micro fiber optic oxygen meter with a 50 µm needle-tip oxygen microsensor, which has a response time of approximately 10 s in liquid media and an accuracy and resolution better than 0.05 kPa. The vial was clamped into a position such that the surface of the media could be viewed with a dissecting microscope mounted for horizontal viewing. The oxygen microsensor was mounted on a Märzhäuser micromanipulator. At the start of the experiment the tip was advanced to about 1 cm above the surface of the media, and then a plug of cotton was placed in the mouth of the vial to reduce air currents within the vial. The tip was advanced toward the media, with P_{O_2} recorded beginning 2 mm above the media surface. The tip was advanced in 0.1 mm increments, with 30 s at each location, until the tip reached approximately 1 cm depth below the media surface, with P_{O_2} recorded continuously. A stable P_{O_2} was reached within a few seconds at each position, and the P_{O_2} at each location was averaged over the entire time that the P_{O_2} reading appeared visually to be stable. In preliminary experiments we found that the electrode reading was minimally affected by the rate of electrode advancement, and the reading reverted quickly when the electrode was pulled back into air, indicating that the electrode retained its sensitivity during the recordings.

Quantification of behavior in anoxia

Eight wandering-stage third instar larvae were weighed and placed individually into a Petri dish filled a few millimeters deep with standard fly media. The Petri dish was covered with plastic film, held in place with a rubber band, and placed under a dissecting scope. The dish was then perfused with normal air (21 kPa P_{O_2}) via tubing that penetrated the wall of the Petri dish; air flowed out of the dish via tubing to an oxygen analyzer (Sable Systems FoxBox). We observed locomotory undulations using the dissecting scope, recorded the time of each undulation using custom-written software, and calculated the proportion of animals moving and the frequency of locomotory undulations per minute over a 10 min period. We then switched the air flowing through the Petri dish to pure nitrogen. Within a few seconds, air exiting the dish had an oxygen concentration of

Journal of Experimental Biology (2015) 218, 2927-2934 doi:10.1242/jeb.125849

less than 0.03 kPa. We recorded the proportion of larvae moving and the frequency of locomotory undulations per minute during 50 min of anoxia.

Adults, 3–4 days old, were tested individually in 1.5 ml chambers fitted with Sable Systems International AD-1 activity detectors that recorded each time the fly moved (Klok et al., 2010). Room air was pushed through the chamber at 100 ml min^{-1}, controlled by a Sable Systems International Flow-Bar8 and then to a LiCOR 6252 CO_2 analyzer. Output of the CO_2 analyzer and AD-1 were recorded at 0.1 Hz. We recorded movements of the flies within the chamber while in room air for a few minutes (Fig. 6). Then the gas flowing through the chamber was switched to nitrogen. Flushing of the chamber with nitrogen was tracked using the rapid drop in CO_2; however, because the volume of the infrared sensor is 11 ml, much larger than the chamber volume, changes in CO_2 as recorded by the LiCOR will lag changes in CO_2 and oxygen in the fly's chamber. The average oxygen level that causes paralysis of *Drosophila* adults is 1 kPa (Klok et al., 2010); thus, we defined the chamber as being anoxic when the O_2 level was less than 1 kPa, 20 ppm CO_2, which would correspond to 20× dilution of the fly's chamber with nitrogen. We calculated equilibration times for the fly's chamber and our flow rate using eqn 8.1 from Lighton (2008); we estimated that a 95% dilution of chamber volume with N_2 would occur in 2.5 s. Therefore, we defined fly exposure to 1 kPa O_2 or less as occurring 2.5 s after the initial sensing of N_2 (indexed by an initial drop in CO_2) by the LiCOR (shown as the left side of the gray box in Fig. 6). Cessation of activity was determined by the absolute difference sums method (Klok et al., 2010; Lighton and Turner, 2004). Briefly, the absolute difference sum of movements of the fly was calculated for each time point, and plotted versus time. A breakpoint in this transformed activity trace showed the time of paralysis (Fig. 6). Time to paralysis after exposure to 1 kPa P_{O_2} or less was calculated for 9 female and 9 male adult flies.

Survival in anoxia

Late third instar larvae were collected and placed with 20–40 individuals in 25×95 mm vials (containing cornmeal–sucrose food). These vials were placed into 1 l near-gas-tight chambers that were perfused with nitrogen throughout the anoxic exposure at a rate sufficient to ensure that P_{O_2} (measured with a downstream Sable Systems FoxBox) was maintained below 0.03 kPa. To reduce the possibility that desiccation might be a cause of death in anoxia trials, the nitrogen stream was bubbled through water, and beakers of water in the anoxia chambers kept relative humidity above 90%, as measured by a Hoboware humidity sensor. After the treatment time, the vials were placed in normoxic conditions, and the number of larvae surviving was assessed as the number of pupae in the vial 24 h later.

Adults were placed 40 animals to a vial and anoxia exposure performed as described for larvae. To ensure precise (±1 s) measurement of time of exposure to anoxia, we measured CO_2 levels in the excurrent air from the chamber with a LiCOR 6252 CO_2 analyzer as the response of this analyzer is very fast (<1 s) relative to our oxygen analyzer; flushing of the vial with nitrogen quickly flushed the CO_2 in the chamber (Fig. 6). Survival was assessed as the number of flies exhibiting movement in response to stimulation 24 h later. Anoxic exposure treatment groups for both larvae and adults were 0.5, 0.75, 1, 1.5, 2, 4, 6, 8 and 12 h. Two to four replicate vials were used for each treatment; 693 larvae and 815 adults were tested. For both life stages, the time at which 50% of animals died (LT50) was calculated by fitting a non-linear model to the survival data.

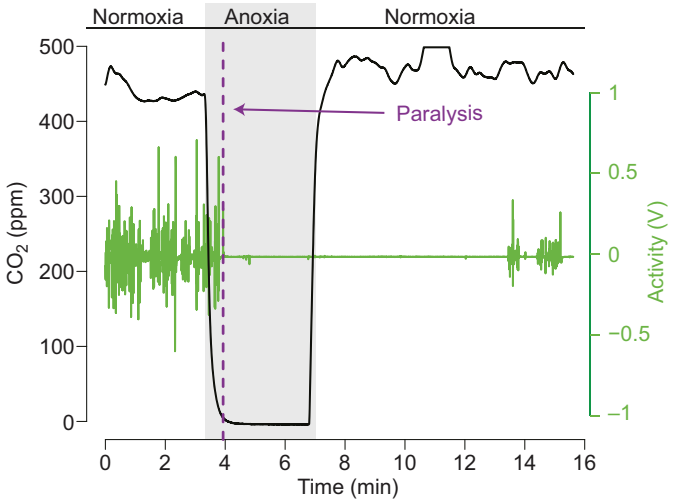

Fig. 6. Time to paralysis of adult flies. In this example, the black line shows CO_2 in the air flowing through the chamber and the green line shows the raw output of the activity detector (technique modified from Lighton and Turner, 2004). The chamber containing the flies was first perfused with room air; the green peaks indicate erratic activity. The shaded box indicates the time during which the normoxic air was switched to nitrogen gas; note the sharp decline in air CO_2 content at the left side of the box, indicating the rapid flushing of the chamber. In under 30 s, activity ceased and remained zero until the perfusion gas was switched back to room air (note revival of activity).

Microcalorimetry

Third instar larvae were reared in normoxic conditions in fly bottles and transported to Louisiana State University for microcalorimetry using methods modified from Hand (1995) and Hoback and Stanley (2001). First, heat dissipation was measured with a thermal activity monitor (model 2277, LKB, Sweden) over 60–120 min of normoxia. Baseline levels without animals were always measured before each experimental run, and often afterwards. Groups of 10–15 late third instar larvae and ∼15, 2–3 day old adults (all males) were placed with a wet disc of paper towel into a 25 ml stainless steel ampoule with two ports, which was perfused with humidified air (21 kPa oxygen) at 200 ml min^{-1} for 3 min and then sealed using a Teflon sealing ring. Adults were transferred into the ampoule without anesthesia by tapping them from their bottle via a funnel into the ampoule. Larvae were counted and weighed before being placed into the ampoule. Vials were handled with gloved hands, and cleaned with ethanol to eliminate water that could evaporate and affect the heat signal. After the normoxic run, the ampoule was removed from the calorimeter, perfused via the ports with humidified argon (0 kPa oxygen) at 200 ml min^{-1} for 3 min, and then reinserted into the calorimeter. Because of the time required for thermal equilibration after reinsertion, the first 20–30 min of data were discarded. In order to avoid anesthetizing the adults, the flies were counted and weighed after calorimetry when they were still paralyzed as a result of the anoxia bout. We successfully completed runs for six groups of adults and four groups of larvae.

Respirometry

Groups of 11–60 late third instar larvae or 2 day old adults were removed from the food without anesthesia and placed into a 5 ml syringe with a wet paper towel to maintain humidity at near-saturation. After the respirometry, adults were anesthetized with CO_2, counted and weighed. Larvae were weighed without anesthesia. Note that for both the respirometry and calorimetry experiments, animals were not post-absorptive, likely elevating our measured metabolic rates over standard rates.

The syringes with animals were flushed with dry, CO_2-free air (Balston Puregas system) for 30 s at 200 ml min^{-1}, which ensured that the syringe had near-zero CO_2 at the beginning of the run, and then sealed and left undisturbed for 55–122 min. A 3 ml bolus was injected from the syringe into plastic tubing and pushed by the pressure generated by the Balston Puregas system at 50 ml min^{-1} through a water scrubber (magnesium perchlorate) and CO_2 scrubber (ascarite), and then the oxygen analyzer (Sable Systems Oxilla). Another 1 ml bolus was injected into a separate airstream and pushed at 200 ml min^{-1} through the LiCor CO_2 analyzer (LI-6252) to measure CO_2 emission rate. The different flow rates and volumes injected into the oxygen and CO_2 analyzers allowed us to match the signal produced by the gas injection to the scale of the instrument. We integrated the area under the peaks produced by the bolus injections and converted this into milliliters of CO_2 produced and O_2 consumed as described in Lighton (2008), converting these data to ml min^{-1} g^{-1} by dividing by the time of syringe closure and the wet mass of *Drosophila* in the syringe. Heat dissipation rates were estimated from oxygen consumption rates, assuming −450 kJ mol^{-1} O_2 for mixed substrate respiration (Hand, 1999).

Journal of Experimental Biology

Lactate assays

Larvae were group-reared in standard bottles in normoxic conditions until the third instar. Late third instar larvae were placed in groups of 2–3 into a 100 ml container with a wet paper towel to ensure full exposure to the test atmosphere without desiccation. Control animals were kept in the container in normoxia for 90 min; we measured lactate levels on the groups of 2–3 larvae after 0 min (larval N=9, adult N=10), 45 min (larval N=5, adult N=10) or 90 min (larval N=6, adult N=10) after placement into the test container. For anoxic exposures, the container was perfused with humidified nitrogen and larvae were collected after 0 h (N=38), 0.13 h (N=15), 0.25 h (N=44), 0.5 h (N=45), 0.75 h (N=45) and 1.33 h (N=9) of anoxia. The same protocol was repeated for 2 day old adults, with 3–4 adults grouped together for each assay (mixture of males and females). Adults were measured after 0 h (N=10), 1.33 h (N=20) and 2.28 h (N=17) of anoxia.

Lactate was measured fluorometrically with an assay modified from Lowry and Passonneau (1972). Animals were ground in a 50× volume of 17.5% perchloric acid and centrifuged for 30 s at 13,000 ***g*** to precipitate proteins with a Marathon 6K centrifuge. A 50 µl aliquot of the supernatant was neutralized using 11 µl of 2 mol l^{-1} KOH, 0.3 mol l^{-1} MOPS, and then centrifuged again for 30 s at 13,000 ***g***. A 10 µl sample of the neutralized supernatant was added to 187 µl of assay buffer formulated so that final concentrations in the assay were 1000 mmol l^{-1} hydrazine, 100 mmol l^{-1} Tris-base, 1.4 mmol l^{-1} EDTA and 2.5 mmol l^{-1} NAD^+ at pH 9.5. A background reading was taken. Then 3 U lactate dehydrogenase (Sigma product number L3916) diluted in 90 µl water was added, and this mixture was allowed to incubate at room temperature for 30 min. NADH levels were measured with fluorescence by exciting samples at 360 nm and measuring emitted fluorescence at 460 nm, using a Standard Curve Filter Fluorometer (Optical Technology Devices, Inc.), correcting for background fluorescence. Lactate concentrations were calculated from standard curves prepared using sodium lactate standards treated identically to the animal extracts.

Acknowledgements

Saman Jirjies quantified larval locomotion.

Competing interests

The authors declare no competing or financial interests.

Author contributions

V.C. collected lactate and respirometry data, helped collect the calorimetry data and contributed to project design, data analysis and manuscript writing. S.C.H. helped collect the calorimetry data and revised the manuscript. J.B.C. collected the mortality and adult behavioral data and edited the manuscript. T.B. collected the media P_{O_2} data. J.F.H. helped collect the calorimetry data, contributed to project design and took leadership of data analysis and manuscript writing.

Funding

This research was partially supported by National Science Foundation (NSF) grants IOS 1122157 and 1256745.

References

Benasayag-Meszaros, R., Risley, M. G., Hernandez, P., Fendrich, M. and Dawson-Scully, K. (2015). Pushing the limit: examining factors that affect anoxia tolerance in a single genotype of adult *D. melanogaster*. *Sci. Rep.* **5**, 9204.

Bennett, A. F. and Gleeson, T. T. (1976). Activity metabolism in the lizard *Scleroporus occidentalis*. *Zoology* **49**, 65-76.

Brust, M. L. and Hoback, W. W. (2009). Hypoxia tolerance in adult and larval *Cicindela* tiger beetles varies by life history but not habitat association. *Ann. Entomol. Soc. Am.* **102**, 462-466.

Cavallaro, M. C. and Hoback, W. W. (2014). Hypoxia tolerance of larvae and pupae of the semi-terrestrial caddisfly (Trichoptera: Limnephilidae). *Ann. Entomol. Soc. Am.* **107**, 1081-1085.

DiGregorio, P. J., Ubersax, J. A. and O'Farrell, P. H. (2001). Hypoxia and nitric oxide induce a rapid, reversible cell cycle arrest of the drosophila syncytial divisions. *J. Biol. Chem.* **276**, 1930-1937.

Doke, J. L., Funk, W. H., Juul, S. T. J. and Moore, B. C. (1995). Habitat availability and benthic invertebrate population changes following alum treatment and hypolimnetic oxygenation in Newman Lake, Washington. *J. Freshwater Ecol.* **10**, 87-102.

Douglas, R. M., Xu, T. and Haddad, G. G. (2001). Cell cycle progression and cell division are sensitive to hypoxia in *Drosophila melanogaster* embryos. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **280**, R1555-R1563.

Douglas, R. M., Farahani, R., Morcillo, P., Kanaan, A., Xu, T. and Haddad, G. G. (2005). Hypoxia induces major effects on cell cycle kinetics and protein expression in *Drosophila melanogaster* embryos. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **288**, R511-R521.

Englisch, H., Opalka, B. and Zebe, E. (1982). The anaerobic metabolism of the larvae of the midge *Chaoborus crystallinus*. *Insect Biochem.* **12**, 149-155.

Feala, J. D., Coquin, L., McCulloch, A. D. and Paternostro, G. (2007). Flexibility in energy metabolism supports hypoxia tolerance in *Drosophila* flight muscle: metabolomic and computational systems analysis. *Mol. Syst. Biol.* **3**, 99.

Feala, J. D., Coquin, L., Zhou, D., Haddad, G. G., Paternostro, G. and McCulloch, A. D. (2009). Metabolism as means for hypoxia adaptation: metabolic profiling and flux balance analysis. *BMC Syst. Biol.* **3**, 91.

Foe, V. E. and Alberts, B. M. (1985). Reversible chromosome condensation induced in *Drosophila* embryos by anoxia: visualization of interphase nuclear organization. *J. Cell Biol.* **100**, 1623-1636.

Gäde, G. (1985). Anaerobic energy metabolism. In *Environmental Physiology and Biochemistry of Insects* (ed. K. H. Hoffmann), pp. 119-136. Berlin: Springer-Verlag.

Galli, G. L. J. and Richards, J. G. (2014). Mitochondria from anoxia-tolerant animals reveal common strategies to survive without oxygen. *J. Comp. Physiol.* **184**, 285-302.

Guppy, M. and Withers, P. (1999). Metabolic depression in animals: physiological perspectives and biochemical generalizations. *Biol. Rev. Camb. Philos. Soc.* **74**, 1-40.

Hand, S. C. (1995). Heat flow is measurable from *Artemia franciscana* embryos under anoxia. *J. Exp. Zool.* **273**, 445-449.

Hand, S. C. (1999). Calorimetric approaches to animal physiology and bioenergetics. In *Handbook of Thermal Analysis and Calorimetry*, Vol. 4, *From Molecules to Man* (ed. R. B. Kemp), pp. 469-510. Amsterdam: Elsevier Science.

Hand, S. C. and Hardewig, I. (1996). Downregulation of cellular metabolism during environmental stress: mechanisms and implications. *Annu. Rev. Physiol.* **58**, 539-563.

Hardewig, I., Addink, A. D. F., Grieshaber, M. K., Portner, H. O. and Van Den Thillart, G. (1991). Metabolic rates at different oxygen levels determined by direct and indirect calorimetry in the oxyconformer *Sipunculus nudus*. *J. Exp. Biol.* **157**, 143-160.

Harrison, J. F. (2015). Handling and use of oxygen by pancrustaceans: conserved patterns and the evolution of respiratory structures. *Integr. Comp. Biol.* (in press), doi:10.1093/icb/icv055.

Harrison, J. F., Philips, J. E. and Gleeson, T. T. (1991). Activity physiology of the two-striped grasshopper, *Melanoplus bivittatus*: gas exchange, hemolymph acid-base status, lactate production and the effect of temperature. *Physiol. Zool.* **64**, 451-472.

Herbert, C. V. and Jackson, D. C. (1985). Temperature effects on the responses to prolonged submergence in the turtle *Chrysemys picta bellii*. II. Metabolic rate, blood acid-base and ionic changes, and cardiovascular function in aerated and anoxic water. *Physiol. Zool.* **58**, 670-681.

Hoback, W. W. and Stanley, D. W. (2001). Insects in hypoxia. *J. Insect Physiol.* **47**, 533-542.

Hoback, W. W., Stanley, D. W., Higley, L. G. and Barnhart, M. C. (1998). Survival of immersion and anoxia by larval tiger beetles, *Cicindela togata*. *Am. Midland Nat.* **140**, 27.

Hoback, W. W., Podrabsky, J. E., Higley, L. G., Stanley, D. W. and Hand, S. C. (2000). Anoxia tolerance of con-familial tiger beetle larvae is associated with differences in energy flow and anaerobiosis. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **170**, 307-314.

Hochachka, P. W., Buck, L. T., Doll, C. J. and Land, S. C. (1996). Unifying theory of hypoxia tolerance: molecular/metabolic defense and rescue mechanisms for surviving oxygen lack. *Proc. Natl. Acad. Sci. USA* **93**, 9493-9498.

Holman, J. D. and Hand, S. C. (2009). Metabolic depression is delayed and mitochondrial impairment averted during prolonged anoxia in the ghost shrimp, *Lepidophthalmus louisianensis* (Schmitt, 1935). *J. Exp. Mar. Biol. Ecol.* **376**, 85-93.

Jackson, D. C. (1997). Lactate accumulation in the shell of the turtle *Chrysemys picta belli* during anoxia at 3C and 10C. *J. Exp. Biol.* **200**, 2295-2300.

Johnston, I. A. and Bernard, L. M. (1983). Utilization of the ethanol pathway in carp following exposure to anoxia. *J. Exp. Biol.* **104**, 73-78.

Keilin, D. and Wang, Y. L. (1946). Haemoglobin of *Gastrophilus* larvae. Purification and properties. *Biochem. J.* **40**, 855-866.

Klok, C. J., Kaiser, A., Lighton, J. R. B. and Harrison, J. F. (2010). Critical oxygen partial pressures and maximal tracheal conductances for *Drosophila melanogaster* reared for multiple generations in hypoxia or hyperoxia. *J. Insect Physiol.* **56**, 461-469.

Kölsch, G., Jakobi, K., Wegener, G. and Braune, H. J. (2002). Energy metabolism and metabolic rate of the alder leaf beetle *Agelastica alni* (L.) (Coleoptera, Chrysomelidae) under aerobic and anaerobic conditions: a microcalorimetric study. *J. Insect Physiol.* **48**, 143-151.

Krishnan, S. N., Sun, Y.-A., Mohsenin, A., Wyman, R. J. and Haddad, G. G. (1997). Behavioral and electrophysiologic responses of *Drosophila melanogaster* to prolonged periods of anoxia. *J. Insect Physiol.* **43**, 203-210.

Lighton, J. R. B. (2008). *Measuring Metabolic Rates: A Manual for Scientists*. New York: Oxford University Press.
Lighton, J. R. B. and Turner, R. J. (2004). Thermolimit respirometry: an objective assessment of critical thermal maxima in two sympatric desert harvester ants, P*ogonomyrmex rugosus* and *P.* californicus. *J. Exp. Biol.* **207**, 1903-1913.
Locasale, J. W. and Cantley, L. C. (2010). Altered metabolsim in cancer. *BMC Biol.* **8**, 88.
Lowry, O. H. and Passonneau, J. V. (1972). *A Flexible System of Enzymatic Analysis*. New York: Academic Press.
Merkey, A. B., Wong, C. K., Hoshizaki, D. K. and Gibbs, A. G. (2011). Energetics of metamorphosis in *Drosophila melanogaster*. *J. Insect Physiol.* **57**, 1437-1445.
Milligan, C. L. and Girard, S. S. (1993). Lactate metabolism in rainbow trout. *J. Exp. Biol.* **180**, 175-193.
Moratzky, T., Burkhardt, G., Weyel, W. and Wegener, G. (1993). Metabolic-rate and tolerance of anoxia-microcalorimetric and biochemical studies on vertebrates and insects. *Thermochim. Acta* **229**, 193-204.
Mueller, W. A., Hassel, M. and Grealy, M. (2015). Metamorphosis and its hormonal control. In *Development and Reproduction in Humans and Animal Specids* (ed. W. A. Mueller, M. Hassel and M. Grealy) pp. 571-584. Berlin: Springer-Verlag.
Oeschger, R. (1990). Long-term anaerobiosis in sublittoral marine invertebrates from the Western Baltic Sea: *Halicryptus spinulosus* (Priapulida), *Astarte borealis* and *Arctica islandica* (Bivalvia). *Mar. Ecol. Prog. Ser.* **59**, 133-143.
Oeschger, R., Peper, H., Graf, G. and Theede, H. (1992). Metabolic responses of *Halicryptus spinulosus* (Priapulida) to reduced oxygen levels and anoxia. *J. Exp. Mar. Biol. Ecol.* **162**, 229-241.
Podrabsky, J. E., Lopez, J. P., Fan, T. W. M., Higashi, R. and Somero, G. N. (2007). Extreme anoxia tolerance in embryos of the annual killifish *Austrofundulus limnaeus:* insights from a metabolomics analysis. *J. Exp. Biol.* **210**, 2253-2266.
Redecker, B. and Zebe, E. (1988). Anaerobic metabolism in aquatic insect larvae: studies on *Chironomus thummi* and *Culex pipiens*. *J. Comp. Physiol. B* **158**, 307-315.
Singer, D. (1999). Neonatal tolerance to hypoxia: a comparative-physiological approach. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **123**, 221-234.
Stieper, B. C., Kupershtok, M., Driscoll, M. V. and Shingleton, A. W. (2008). Imaginal discs regulate developmental timing in *Drosophila melanogaster*. *Dev. Biol.* **321**, 18-26.
Tennessen, J. M., Baker, K. D., Lam, G., Evans, J. and Thummel, C. S. (2011). The Drosophila estrogen-related receptor directs a metabolic switch that supports developmental growth. *Cell Metab.* **13**, 139-148.
Theede, H. (1984). Physiological approaches to biological problems of the Baltic. *Limnologica* **15**, 443-458.
Theede, H., Ponat, A., Hiroki, K. and Schlieper, C. (1969). Studies on the resistance of marine bottom invertebrates to oxygen-deficiency and hydrogen sulphide. *Mar. Biol.* **2**, 325-337.
van Ginneken, V. and van den Thillart, G. (2009). Metabolic depression in fish measured by direct calorimetry: a review. *Thermochim. Acta* **483**, 1-7.
Vander Heiden, M. G., Cantley, L. C. and Thompson, C. B. (2009). Understanding the Warburg Effect: the metabolic requirements of cell proliferation. *Science* **324**, 1029-1033.
Vigne, P., Tauc, M. and Frelin, C. (2009). Strong dietary restrictions protect *Drosophila* against anoxia/reoxygenation injuries. *PLoS ONE* **4**, e5422.
Wegener, G. (1987). Insect brain metabolism under hypoxic and normoxic conditions. In *Arthropod Brain: Its Evolution*, *Development*, *Structure*, and *Function* (ed. A. Gupta), pp. 369-397. New York: John Wiley and Sons.
Wegener, G. (1988). Oxygen availability, energy metabolism, and metabolic rate in invertebrates and vertebrates. In *Oxygen Sensing in Tissues* (ed. H. Acker), pp. 13-35. Berlin: Springer-Verlag.
Wingrove, J. A. and O'Farrell, P. H. (1999). Nitric oxide contributes to behavioral, cellular, and developmental responses to low oxygen in *Drosophila*. *Cell* **98**, 105-114.
Wyatt, T. D. (1986). How a subsocial intertidal beetle, Bledius spectabilis, prevents flooding and anoxia in its burrow. *Behav. Ecol. Sociobiol.* **19**, 323-331.

Journal of Experimental Biology